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Frizzled3 Expression and Colony Development in Hydractiniid Hydrozoans

Running title: *Frizzled3* and Coloniality

S.M. Sanders*, M.K. Travert and P. Cartwright[#]

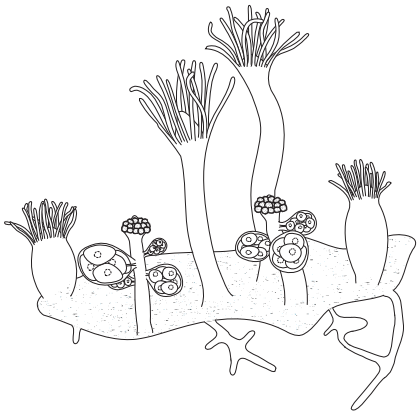
Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence KS USA

*Present address: Thomas E. Starzl Transplantation Institute and Center for Evolutionary Biology and Medicine, University of Pittsburgh, Pittsburgh, PA, USA

[#]Corresponding Author: 1200 Sunnyside Ave, Lawrence KS 22045: Email pcart@ku.edu

Research Highlights

Frizzled3 expression in two colonial hydrozoans is consistent with it playing a role in the development and patterning of colony-specific tissues, implicating the Wnt signaling pathway in the evolution and development of hydrozoan colonies.



Keywords: Hydrozoa, coloniality, Wnt pathway, *Frizzled3*

Abstract

Hydractiniid hydrozoan colonies are comprised of individual polyps connected by tube-like stolons or a sheet-like mat. Mat and stolons function to integrate the colony through continuous epithelia and shared gastrovascular cavity. Although mechanisms of hydrozoan polyp development have been well studied, little is known about the signaling processes governing the patterning of colonies. Here we investigate the Wnt receptor family Frizzled. Phylogenetic analysis reveals that hydrozoans possess four Frizzled orthologs. We find that one of these genes, *Frizzled3,* displays a spatially restricted expression pattern in colony specific tissue in two hydractiniid hydrozoans, *Hydractinia symbiolongicarpus* and *Podocoryna carnea*, in a manner that corresponds to their distinct colony forms (stolonal mat in *Hydractinia* and free stolons in *Podocoryna*). Interestingly, *Frizzled3* was lost in the genome of *Hydra*, which is a solitary polyp and thus lacks colony specific tissue. Current evidence suggests that the Wnt signaling pathway plays a key role in the evolution of colony diversity and colony loss in Hydrozoa.

Introduction

Hydrozoans are known for their complex life cycles that, in most species, include a benthic colonial stage. Colonial hydrozoans are composed of individual polyps connected by continuous epithelia, forming the shared gastrovascular cavity. In hydractiniid hydrozoans, the structures connecting the polyps are sheet-like mat tissue or elongating tube-like stolons. The stolons grow outward from the primary polyp and bud more polyps to form a colony (Supplemental Figure 1).

Although considerable insight has been gained in the patterning of hydrozoan polyps, much less is known about the developmental mechanisms underlying colony formation (although see Cartwright et al., 2006, Hensel et al., 2014, and Bagaeva et al., 2019). The Wnt pathway is a good candidate given that it plays a prominent role in patterning the polyp (Hobmayer et al., 2000; Momose and Houliston, 2007; Müller et al., 2007; Plickert et al., 2006), medusa (Momose and Houliston, 2007; Nawrocki and Cartwright, 2013; Sanders and Cartwright, 2015a), and more recently, colony form (Hensel et al., 2014; Bagaeva et al., 2019). Here we implicate the Wnt receptor *Frizzled3* in the development of colony specific structures in two hydractiniid hydrozoans.

Materials and Methods

Molecular Phylogenetic Analyses. Cnidarian sequences belonging to the Frizzled gene family with a seven transmembrane domain were mined from NCBI's non-redundant (nr) protein database and used to probe published cnidarian transcriptomes and genomes (Supplemental Table 1) for members of the Frizzled gene family using BLAST. Many of the genome and transcriptome sequences were from NCBI short read archive (SRA) and needed to be assembled before mining for Frizzled genes. Prior to assembly, each library was trimmed of low quality reads and adapters using fastp (Chen et al., 2018). For those transcriptomes consisting of different libraries, filtered reads were combined into a single dataset, subsequently *de novo* transcriptome assembly was carried out using Trinity v2.8.5 (Grabherr et al., 2011). Sequences with significant alignments to published cnidarian Frizzled proteins (>100 amino acids in length) were extracted from the assemblies, translated using EMBOSS (Rice et al. 2000), and redundant gene copies were removed with CD-HIT (Li and Godzik, 2006). To confirm these sequences as

Frizzled genes, each sequence was annotated using HMMscan (http://hmmer.org/) and checked for significant alignments (e-value ≤ 1e-10) to the cysteine-rich (CRD, Pfam PF01392) and seven-pass transmembrane Frizzled domain (Fz-7tm, Pfam PF01534). Sequences without the Fz-7tm domain were excluded from further analysis.

Remaining sequences were then aligned with MAFFT (Katoh et al. 2005) using the L-insi alignment algorithm and ambiguously aligned regions were removed from the alignment with Gblocks (Castresana, 2000). A maximum likelihood estimate of the Frizzled gene tree was inferred using RAxML (Stamatakis et al. 2008) on the CIPRES portal (Miller et al. 2010). Support was assessed using the rapid bootstrapping algorithm (-f a) with 1,000 bootstrap replicates under the PROTGAMMA+WAG model. Alignments and tree files can be found in supplemental data.

Animal Care. Animal care was performed as in Sanders and Cartwright (2015b). For *in situ* hybridizations young colonies were reared and fixed for downstream analysis. *H. symbiolongicarpus*, young colonies were generated by inducing spawning by light after keeping animals in the dark for ~48 hours. For *P. carnea*, freshly liberated medusae were collected and fed one-day-old nauplii of *Artemia* and then spawning was induced by light after keeping medusae in the dark for ~24 hours. Eggs and sperm were collected and kept in a Petri dish for ~72 hours. By day three, metamorphosis-competent larvae in both species were incubated for three hours in 116mM CsCl in seawater (Frank et al. 2001). *P. carnea* stolon regeneration was induced by excising gastrozooids from the colony and kept in Petri dishes on glass coverslips. Prior to tissue collection for whole mount *in situ* hybridization, specimen were starved for three

days, anesthetized with menthol crystals, and fixed in 4% paraformaldehyde overnight at 4°C. Fixed tissue was rinsed with and stored in 100% methanol at -20°C.

Probe Synthesis and *in situ* Hybridization. Sequences for *Frizzled3* transcripts were identified from previously published transcriptome assemblies of *H. symbiolongicarpus* (Sanders et al. 2014) and *P. carnea* (Sanders and Cartwright, 2015a, b). *Frizzled3* was amplified from each species cDNA using the following PCR primers: *H. symbiolongicarpus* forward 5′-TTTGTCGCACTTCCTCTGCT-3′ and reverse 5′-TCCGCTAGTCACACCTACGA-3′ to obtain a 351bp fragment; *P. carnea* forward 5′-TGGTATGGCATCCGCACTTT-3′ and reverse 5′-CCAACAACAACCGAAGCTGG-3′ to obtain a 420bp fragment. These products were cloned using the Invitrogen TOPO-TA Cloning Kit and DIG labeled riboprobes were synthesized from clones using the Invitrogen T7/T3 Megascript kit. ISH protocol was adapted from Gajewsky et al. (1996). Hybridization was carried out at 50˚C for 16-18 hours with a probe concentration of 0.1 ng/µl. Hybridization was detected by immunostaining with anti-DIG-Fab-AP (ROCHE) and NBT/BCIP.

Results

Molecular Phylogeny of Cnidarian Frizzleds. In an effort to clarify the role of the Wnt pathway in colony ontogeny, we focused our attention on the Wnt receptor gene family Frizzled. Frizzled genes encode proteins with a unique seven-pass transmembrane domain (Fz-7tm) and an extracellular Wnt binding domain (Bhanot et al., 1996). Phylogenetic analysis of Frizzled genes from 55 medusozoan genomes and/or transcriptomes (Supplemental Table 1) revealed that hydrozoans possess four Frizzled orthologs (Figure 1) with several lineage specific duplications

(denoted by asterisks). Notably, *Frizzled3* is completely absent from all three *Hydra* genomes sampled (Supplemental Table 2), suggesting that that loss of coloniality and colony specific-tissues in the *Hydra* lineage coincides with the loss of *Frizzled3.*

A close relative of *Hydra*, *Ectopleura larynx* is technically colonial, but their colonies form primarily from fusion of sexually reproduced juvenile polyps (Nawrocki and Cartwright, 2012; Nawrocki et al., 2013; Chang et al., 2018). Unlike *Hydra*, *Ectopleura* retains colony-specific tissue aboral to the polyp that it uses to build the chimeric colony (Nawrocki and Cartwright, 2012). The assertion that the *Ectopleura* stalk is colonial tissue and not polyp tissue is supported by the expression of the gene *manacle*, which in *Hydra*, marks the base of the polyp (Bridge et al., 2000) and in *Ectopleura* marks the transition between the aboral whorl of tentacles in the polyp and the stalk (Nawrocki and Cartwright, 2012). *Ectopleura* has the *Frizzled3* gene, consistent with its retention of colony specific tissue. These findings indicate that *Frizzled3* is correlated with the presence of colony-specific tissues. It should be noted that this correlation appears to be specific to the hydrozoan clade Hydroidolina, as *Frizzled3* was found in holopelagic (lacking a polyp) and solitary members of trachyline hydrozoans (Supplemental Table 2).

***Frizzled3* Expression in Two Colonial Cnidarians.** Given this apparent correlation of coloniality and the presence of the *Frizzled3* gene, we hypothesized that *Frizzled3* is involved in colony patterning in hydrozoans. Thus, we documented the spatial expression of *Frizzled3* during early colony development of two hydractiniid hydrozoans *H. symbiolongicarpus* and *P. carnea* with whole mount *in situ* hybridization methods (ISH).

Although closely related, these two species display significant differences in colony form. Mature colonies of *Hydractinia* develop a stolonal mat in addition to their tube-shaped branching stolons (Supplemental Figure 1C). The stolonal mat is characterized by an epithelial sheet covering gastrovascular canals. The stolonal mat has been shown to be histologically and developmentally distinct from the peripheral stolons (Cartwright and Buss, 1999). By contrast, *P. carnea* lacks a stolonal mat and its colony is comprised exclusively of a branching network of tube-like stolons.

In *H. symbiolongicarpus*, *Frizzled3* expression was detected in stolon tissues 24 hours after the induction of metamorphosis (Fig. 2A-C). *Frizzled3* expression at this stage was specific to and widespread in the epidermis of the more proximal portions of the newly formed stolons, and was excluded from the stolon tips and polyp base (Fig. 2A, B). This pattern continues throughout colony ontogeny. In mature colonies of *H. symbiolongicarpus*, *Frizzled3* expression remains primarily restricted to the stolon tissue (Fig. 2D, E), but more distal portions of the stolons show a more discontiguous expression pattern (Fig. 2E), in what appears to be stem cells called interstitial cells (*i*-cells) that are migrating along the stolons. As colony expansion continues, branched stolons anastomose and form the continuous epidermal mat. Correspondingly, the *Frizzled3* expression boundary along the epidermal edge of the stolons is eliminated (Fig. 2D). In non-stoloniferous colonies, accumulated expression can be seen around the periphery of the mat (Fig. 2F, G). Furthermore, there are clusters of epithelial cells expressing *Frizzled3* within the mat tissues near the edge of the colony (Fig. 2G).

Although *Podocoryna* does not have a stolonal mat, similar expression patterns were observed in the stolons emanating from the primary polyp (Fig. 3A) and in mature, stoloniferous colonies (not shown). During stolon regeneration in *P. carnea*, *Frizzled3* expression was

examined in polyps at 24, 48, and 72 hours after excision from the colony. At 24 hours post excision, stolons had not formed and *Frizzled3* expression was not detected (not shown). By 48 hours, stolons had regenerated and ISH revealed *Frizzled3* expression in the epidermis of the regenerated stolons (Fig. 3B) and continued in 72 hour regenerated stolons (Fig. 3C). Similar to expression in stolons of *H. symbiolongicarpus*, *Frizzled3* expression was restricted to the proximal portion of the stolons and excluded from the stolon tips throughout all stages of stolon regeneration (Fig. 3B). Similar expression of *Frizzled3* during normal development and regeneration suggests the same pathways are regulating both of these processes.

Discussion

While *Frizzled3* expression has been observed in other hydrozoans, it has not yet been explored in the context of colony ontogeny. Previous studies have reported *Frizzled3*'s role in embryology (Momose and Houliston, 2007; Momose et al., 2008; Amiel and Houliston, 2009), polyp patterning (Momose and Houliston, 2007; Sanders et al., 2014, Sanders and Cartwright, 2015a), and medusae development (Momose and Houliston, 2007; Sanders and Cartwright, 2015a). In the leptothecate hydrozoan *Clytia hemisphaerica*, *Frizzled3* expression appears to maintain the aboral identity of the embryo (Momose and Houliston, 2007; Momose et al., 2008; Amiel and Houliston, 2009), yet is expressed at the oral ends of both the polyp and medusae (Momose and Houliston, 2007). Similarly, in *P. carnea*, *Frizzled3* is expressed at the tip of the hypostome (mouth) of the polyp and at the oral end of the medusae throughout development (Sanders and Cartwright, 2015a).

These pleiotropic expression patterns of *Frizzled3* in polyp, medusae, and colony patterning suggest repeated co-option of this gene in different developmental contexts to mediate

different Wnt ligands. Given that there are 10 hydrozoan Wnt orthologs (Hensel et al., 2014) and only four membrane-bound Wnt receptors (Frizzleds) (Figure 1) to transduce the Wnt signal into the cell, it is not surprising that Frizzled receptors are acting in multiple contexts.

While there has been no study showing a specific Wnt ligand's role in stolon regeneration and development, there is considerable evidence that different Wnt ligands (and their Frizzled receptors) act antagonistically to pattern oral and aboral structures of the polyp. Proper aboral patterning involves the growth of stolons from the aboral end of the polyp in encrusting colonial forms. In *H. echinata* ectopic induction of canonical Wnt signaling (which signals oral structures) during regeneration, prevents stolon development during metamorphosis (Duffy et al., 2010). *Wnt16*, *Wnt11a*, and *Wnt2* are expressed in the stolons of *H. echinata* (Hensel et al., 2014). Ectopic induction of canonical Wnt signaling with azakenpaullone treatments shows that canonical Wnt signaling up-regulates *Wnt2* expression while down-regulating *Wnt11a* expression in the stolons (Hensel et al., 2014), further suggesting that *Wnt11a* plays a role in patterning of the aboral end, including stolon budding. Our findings on *Frizzled3* expression, combined with the findings of Hensel et al. (2014), suggests that *Frizzled3* is likely the receptor of *Wnt11a* and these two genes are at least partly responsible for the development and growth of the colony.

In addition, our comparisons of expression between *Hydractinia* and *Podocoryna* suggest that *Frizzled3* may play a role in distinct colony-specific tissue,s the stolonal mat and stolons. This evidence is further underscored by the findings that *Frizzled3* and *Wnt11a* (see Hensel et al., 2014) have been lost in *Hydra*, which is solitary and does not bud stolons (Figure 1 and Supplemental Table 2). While the absence of these genes in the solitary *Hydra* lineage is compelling, it is important to note that the link between *Frizzled3* and evolution of coloniality

awaits confirmation through further sampling of other hydrozoan lineages that have lost coloniality to determine if there are repeated correlations between the loss of coloniality and the loss of *Frizzled3* and *Wnt11a* genes.

Conclusions

Our phylogenetic analysis of the Frizzled gene family recovered four Frizzled orthologs in hydrozoans and that *Frizzled3* was lost in the solitary *Hydra* (Figure 1 and Supplemental Table 2). *Frizzled3* expression patterns in developing colonies of *H. symbiolongicarpus* and *P. carnea* are consistent with it playing a role in regulating colony patterning and growth as they are expressed in a spatially restricted manner in colony specific tissues, although conclusive evidence awaits functional experimentation. The differences in expression also correspond to differences in colony specific tissues, as *Frizzled3* is expressed at the periphery of the epithelia mat in *Hydractinia* and in the proximal regions of stolons in both *Hydractinia* and *Podocoryna*. Moreover, a study of Wnt genes in *H. echinata* found compelling evidence that *Wnt11a* might be a Wnt ligand regulating stolon growth (Hensel et al., 2014) and act antagonistically to the canonical Wnt pathway patterning the polyp. Together, existing evidence suggests that coloniality in hydrozoans was likely accompanied by co-option and subfunctionalization of certain Wnt ligands and Frizzled receptors to direct patterning of colonies, although further studies with increased taxon sampling and functional experiments are needed corroborate this hypothesis.

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Figure Legends

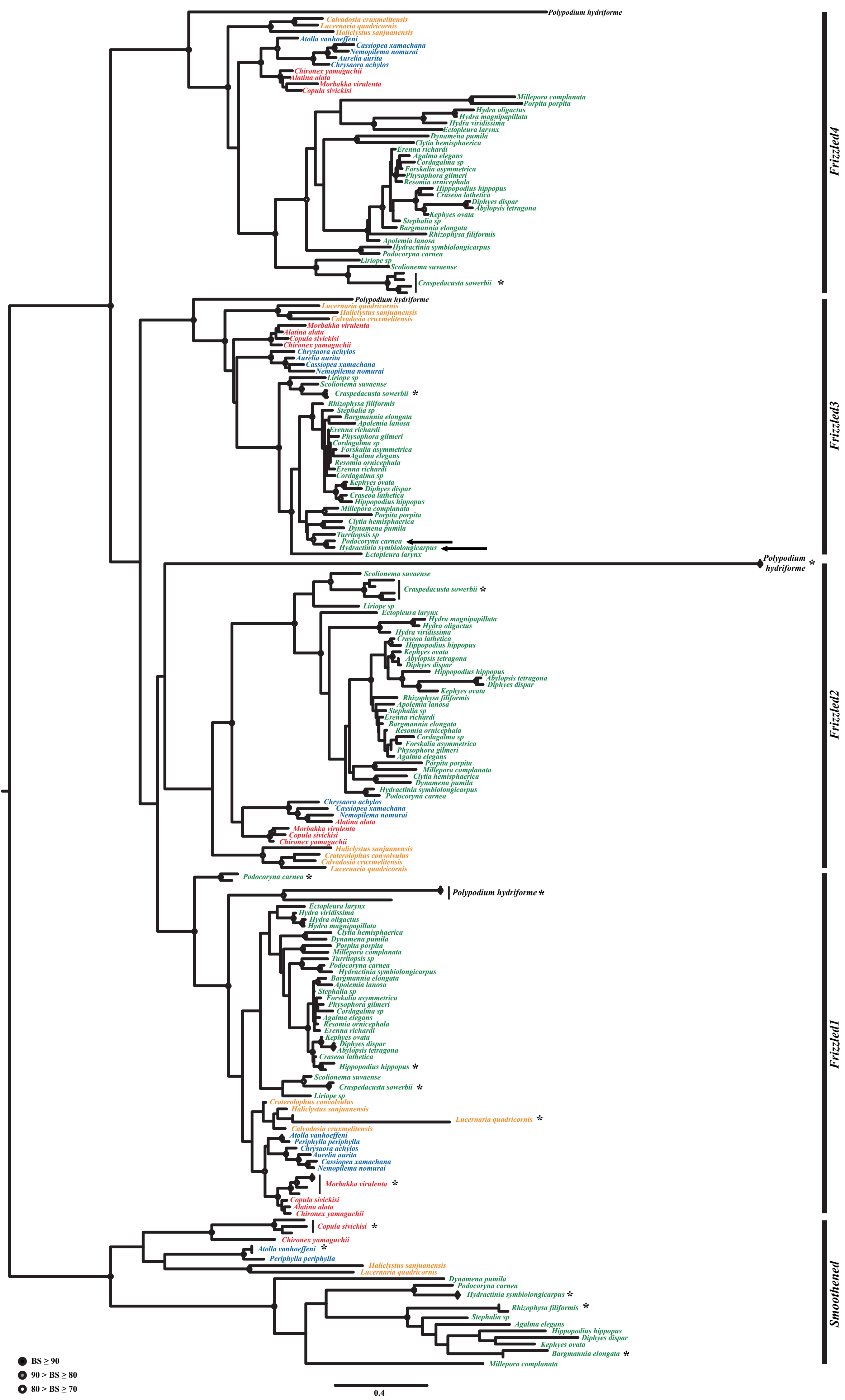
Figure 1. Phylogenetic analysis of medusozoan Frizzled genes. Maximum likelihood (ML) estimate of the medusozoan Frizzled gene tree recovers four well supported medusozoan

Frizzled orthologs: *Frizzled1, Frizzled2, Frizzled3,* and *Frizzled4*. ML bootstrap support values not shown for nodes with less than 70% support. The tree is rooted on medusozoan Smoothened genes (which have a similar Fz-7tm domain, but lack the CRD domain) that were recovered by the Frizzled gene survey. Arrows mark the *Frizzled3* genes selected for ISH. Asterisks indicate lineage specific Frizzled genes duplications. Tips are labeled by species and color coded by class: Black = Myxozoa; Orange = Staurozoa; Blue = Scyphozoa; Red = Cubozoa; Green = Hydrozoa.

Figure 2. Whole mount *in situ* hybridization of *Frizzled3* in *H. symbiolongicarpus.*
A) Aboral view of primary polyp and newly budded stolons 24 hours after induction of metamorphosis showing *Frizzled3* expression in proximal region of stolons (in blue). B) Lateral view of primary polyp and newly budded stolons 24 hours after induction of metamorphosis. C) Aboral view of a primary polyp and stolons 48 hours after induction of metamorphosis showing *Frizzled3* expression throughout stolons. D) Stoloniferous colony showing *Frizzled3* expression in the epidermis at the edges of the mat and stolons. E) Higher magnification of stolons more distal to the colony. F) Mat tissue showing expression at the distal ends. G) Higher magnification of colony mat. Legend: i – i-cells; ec – epithelial cell clusters; pp – primary polyp; p – polyps; t – stolon tip. Arrows indicate areas of accumulated expression along the edge of the mat. Arrowheads mark regions where the epidermis is fusing over stolons, forming a continuous epidermal mat. Dashed line in panel D roughly marks the boundary between the mat and peripheral stolons.

Figure 3. Whole mount *in situ* hybridization of *Frizzled3* in *P. carnea*.

A) Oral view of a primary polyp and newly budded stolons 24 hours after induction of metamorphosis showing *Frizzled3* expression (in blue) at the proximal region of the stolons. B) Regenerating stolons 48 hours after removal from the colony. C) Regenerating stolon 72 hours after removal from the colony. Legend: pp – primary polyp; t – stolon tip.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Lucernaria quadricornis
Haliclystus sanjuanensis
Atolla vanhoeffeni
Cassiopea xamachana
Nemopilema nomurai
Aurelia aurita
Chrysaora achylos
Chironex yamaguchii
Alatina alata
Morbakka virulenta
Copula sivickisi
Millepora complanata
Porpita porpita
Hydra oligactus
Hydra magnipapillata
Hydra viridissima
Ectopleura larynx
Dynamena pumila
Clytia hemisphaerica
Erenna richardi
Agalma elegans
Cordagalma sp
Forskalia asymmetrica
Physophora gilmeri
Resomia ornicephala
Hippopodius hippopus
Craseoa lathetica
Diphyes dispar
Abylopsis tetragona
Kephyes ovata
Stephalia sp
Bargmannia elongata
Rhizophysa filiformis
Apolemia lanosa
Hydractinia symbiolongicarpus
Podocoryna carnea
Liriope sp
Scolionema suvaense
Craspedacusta sowerbii *
Frizzled4
Polypodium hydriforme
Lucernaria quadricornis
Haliclystus sanjuanensis
Calvadosia cruxmelitensis
Morbakka virulenta
Alatina alata
Copula sivickisi
Chironex yamaguchii
Chrysaora achylos
Aurelia aurita
Cassiopea xamachana
Nemopilema nomurai
Liriope sp
Scolionema suvaense
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Bargmannia elongata
Apolemia lanosa
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Physophora gilmeri
Cordagalma sp
Forskalia asymmetrica
Agalma elegans
Resomia ornicephala
Erenna richardi
Cordagalma sp
Kephyes ovata
Diphyes dispar
Craseoa lathetica
Hippopodius hippopus
Millepora complanata
Porpita porpita
Clytia hemisphaerica
Dynamena pumila
Turritopsis sp
Podocoryna carnea
Hydractinia symbiolongicarpus
Ectopleura larynx
Frizzled3
Polypodium hydriforme *
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Ectopleura larynx
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Hydra oligactus
Hydra viridissima
Craseoa lathetica
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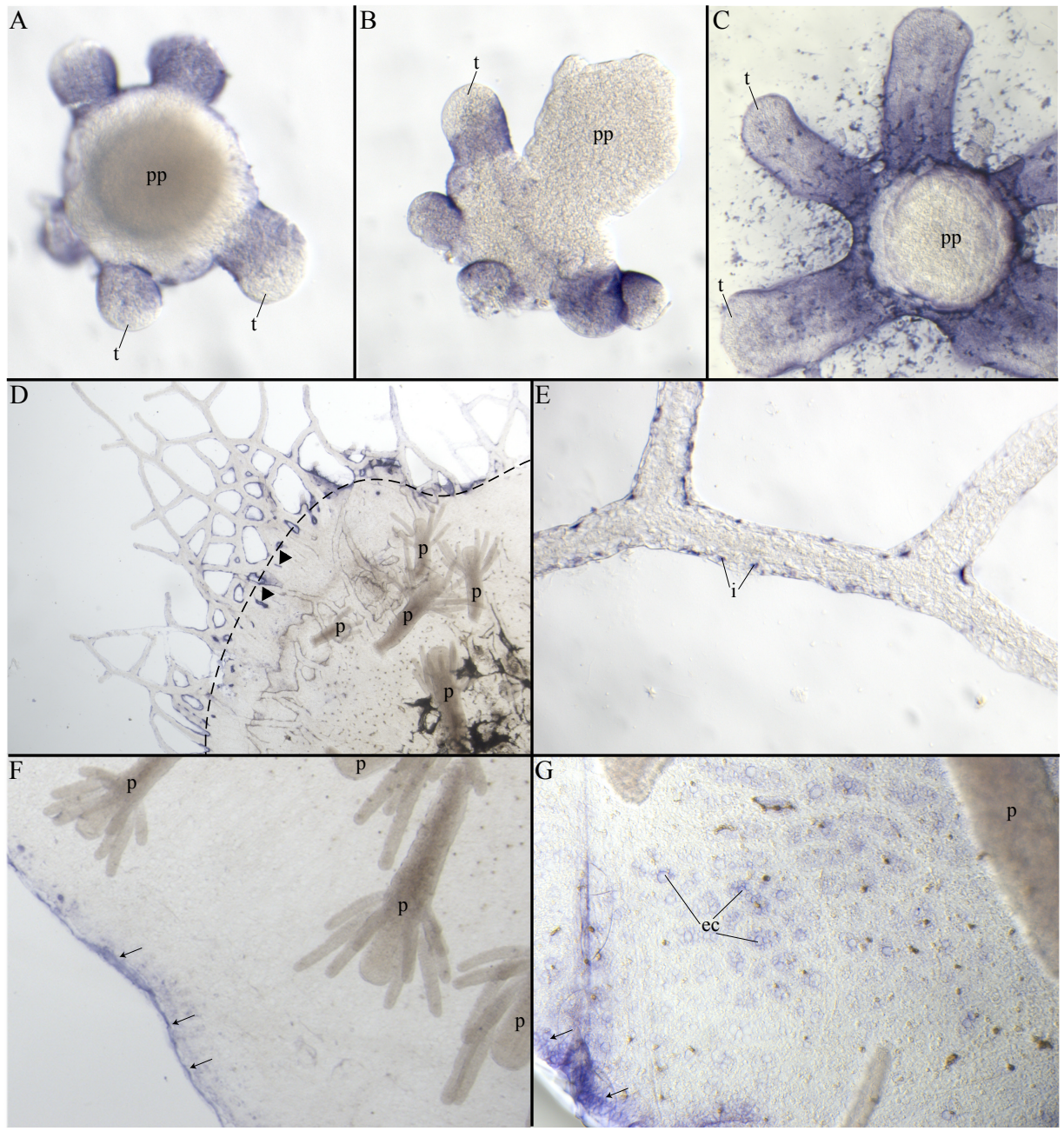
Resomia ornicephala
Cordagalma sp
Forskalia asymmetrica
Physophora gilmeri
Agalma elegans
Porpita porpita
Millepora complanata
Clytia hemisphaerica
Dynamena pumila
Hydractinia symbiolongicarpus
Podocoryna carnea
Chrysaora achylos
Cassiopea xamachana
Nemopilema nomurai
Alatina alata
Morbakka virulenta
Copula sivickisi
Chironex yamaguchii
Haliclystus sanjuanensis
Craterolophus convolvulus
Calvadosia cruxmelitensis
Lucernaria quadricornis
Frizzled2
Podocoryna carnea *
Polypodium hydriforme *
Ectopleura larynx
Hydra viridissima
Hydra oligactus
Hydra magnipapillata
Clytia hemisphaerica
Dynamena pumila
Porpita porpita
Millepora complanata
Turritopsis sp
Podocoryna carnea
Hydractinia symbiolongicarpus
Bargmannia elongata
Apolemia lanosa
Stephalia sp
Forskalia asymmetrica
Physophora gilmeri
Cordagalma sp
Agalma elegans
Resomia ornicephala
Erenna richardi
Kephyes ovata
Diphyes dispar
Abylopsis tetragona
Craseoa lathetica
Hippopodius hippopus *
Scolionema suvaense
Craspedacusta sowerbii *
Liriope sp
Craterolophus convolvulus
Haliclystus sanjuanensis
Lucernaria quadricornis *
Calvadosia cruxmelitensis
Atolla vanhoeffeni
Periphylla periphylla
Chrysaora achylos
Aurelia aurita
Cassiopea xamachana
Nemopilema nomurai
Morbakka virulenta *
Copula sivickisi
Alatina alata
Chironex yamaguchii
Frizzled1
Copula sivickisi *
Chironex yamaguchii
Atolla vanhoeffeni *
Periphylla periphylla
Haliclystus sanjuanensis
Lucernaria quadricornis
Dynamena pumila
Podocoryna carnea
Hydractinia symbiolongicarpus *
Rhizophysa filiformis *
Stephalia sp
Agalma elegans
Hippopodius hippopus
Diphyes dispar
Kephyes ovata
Bargmannia elongata *
Millepora complanata
Smoothened
BS ≥ 90
90 > BS ≥ 80
80 > BS ≥ 70
0.4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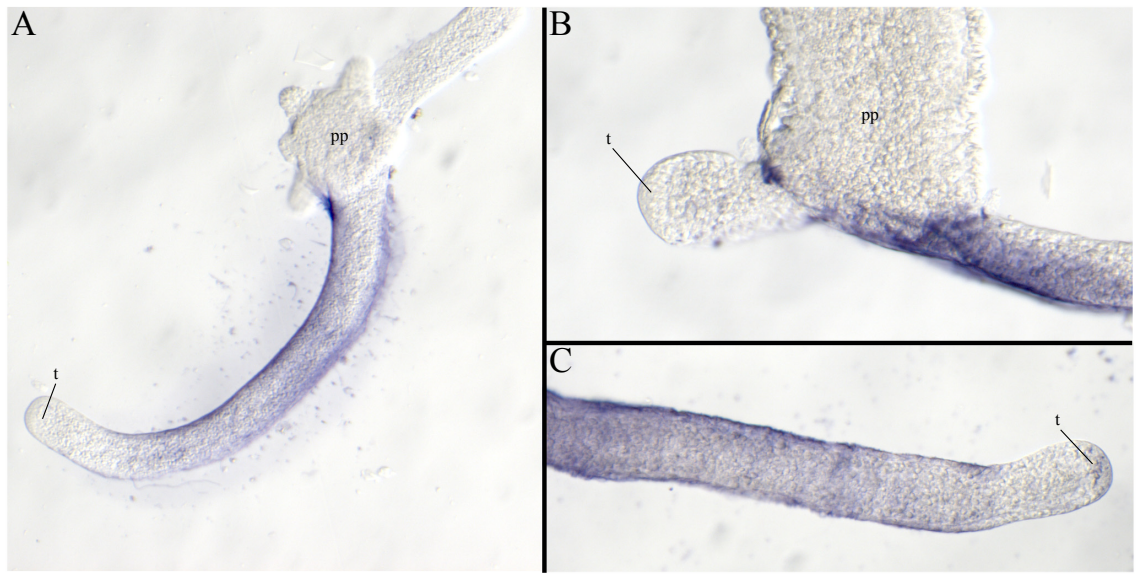
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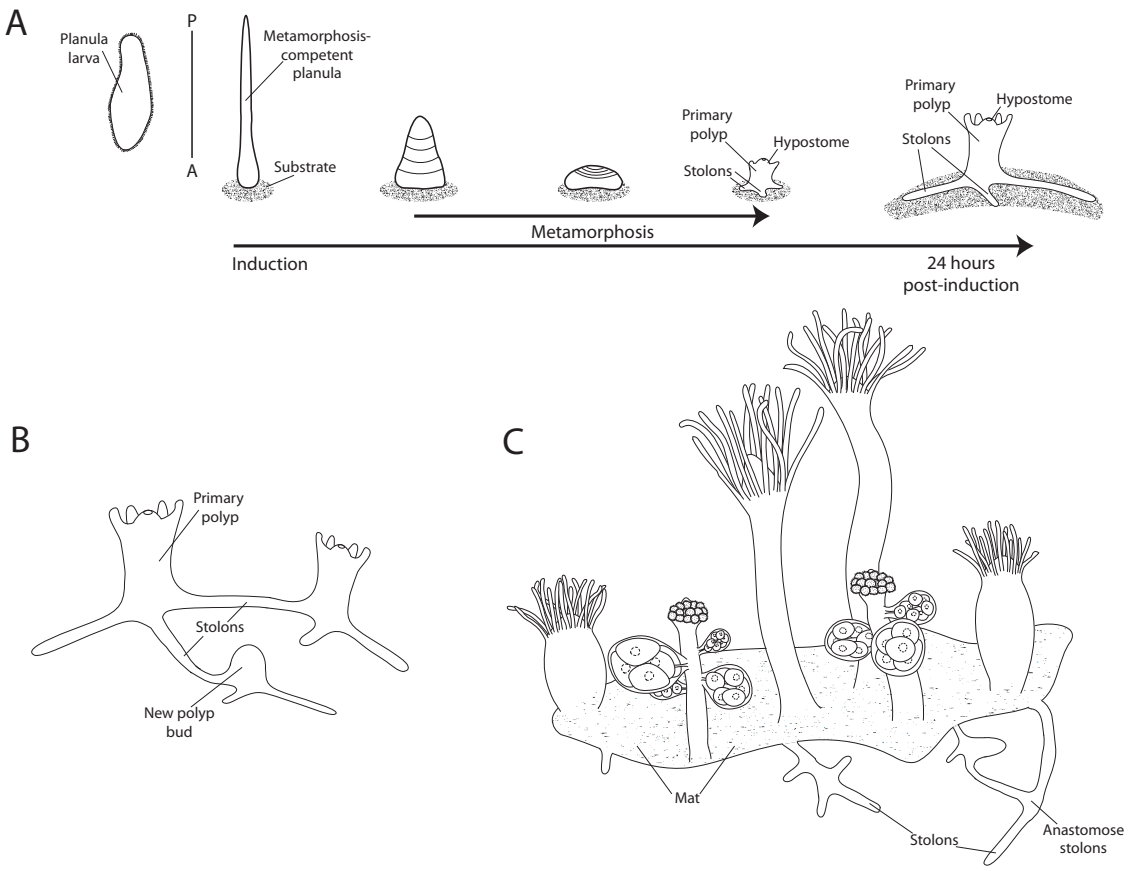
SUPPORTING INFORMATION



Supplemental Figure 1. *Hydractinia symbiolongicarpus* life cycle.

A) Illustration of metamorphosis in *H. symbiolongicarpus.* About three days after fertilization, the anterior end (labeled 'A') of a metamorphosis-competent planula will attach to the substrate and is able to metamorphose into a polyp. Metamorphosis is induced with a three hour incubation in 116mM CsCl. During metamorphosis, the posterior end (labeled 'P') of the planula compresses and forms concentric rings that will become the hypostome of the polyp. The oral end will then begin to expand and form the hypostome, mouth and tentacles. As this occurs, stolons also begin to branch from the polyp base and by 24 hours the primary polyp is fully

developed with branching stolons. B) As stolons continue to branch and elongate, new polyps are produced and form a colony. C). Over time the colony matures and a central continuous epithelial mat forms, from which peripheral stolons can grow, branch, and anastomose.

Supplemental Table 1. Genome and transcriptome data used for identification of *Frizzled* genes.

Species	Class	Subclass	Order	Family	Assembly Type	Accession
Nematostella vectensis	Anthozoa	Hexacorallia	Actiniaria	Edwardsiidae	Genome	GCA_000209225.1
Corynactis australis	Anthozoa	Hexacorallia	Corallimorpharia	Corallimorphidae	Transcriptome	GELM01
Acropora digitifera	Anthozoa	Hexacorallia	Scleractinia	Acroporidae	Genome	GCA_000222465.2
Zoanthus sp. QL-2018	Anthozoa	Hexacorallia	Zoantharia	Zoanthidae	Transcriptome	GGTW01
Dendronephthya gigantea	Anthozoa	Octocorallia	Alcyonacea	Nephtheidae	Genome	GCA_004324835.1
Heliopora coerulea	Anthozoa	Octocorallia	Helioporacea	Helioporidae	Transcriptome	GFVH01; IABP01
Renilla reniformis	Anthozoa	Octocorallia	Pennatulacea	Renillidae	Genome	GCA_900177555.1
Alatina alata	Cubozoa	N/A	Carybdeida	Alatinidae	Transcriptome	GEUJ01
Morbakka virulenta	Cubozoa	N/A	Carybdeida	Carukiidae	Genome	GCA_003991215.1
Copula sivickisi	Cubozoa	N/A	Carybdeida	Tripedaliidae	Transcriptome	GHBG01
Chironex yamaguchii	Cubozoa	N/A	Chirodropida	Chirodropidae	Transcriptome	GHAX01
Hydractinia symbiolongicarpus	Hydrozoa	Hydroidolina	Anthoathecata	Hydractiniidae	Transcriptome	GAWH01; GCHW01
Podocoryna carnea	Hydrozoa	Hydroidolina	Anthoathecata	Hydractiniidae	Transcriptome	GBEH01; GCHV01
Hydra oligactis	Hydrozoa	Hydroidolina	Anthoathecata	Hydridae	Genome	GCA_004118135.1
Hydra viridissima	Hydrozoa	Hydroidolina	Anthoathecata	Hydridae	Genome	GCA_004118115.1
Hydra magnipapillata	Hydrozoa	Hydroidolina	Anthoathecata	Hydridae	Genome	GCA_000004095.1
Millepora complanata	Hydrozoa	Hydroidolina	Anthoathecata	Milleporidae	Transcriptome	GFGT01
Turritopsis sp. SK-2016	Hydrozoa	Hydroidolina	Anthoathecata	Oceaniidae	Transcriptome	IAAF01
Porpita porpita	Hydrozoa	Hydroidolina	Anthoathecata	Porpitidae	Transcriptome	GHBA01
Ectopleura larynx	Hydrozoa	Hydroidolina	Anthoathecata	Tubulariidae	Transcriptome	SRR923510
Clytia hemisphaerica	Hydrozoa	Hydroidolina	Leptothecata	Campanulariidae	Genome	GCA_902728285.1
Dynamena pumila	Hydrozoa	Hydroidolina	Leptothecata	Sertulariidae	Transcriptome	GHMC01
Abylopsis tetragona	Hydrozoa	Hydroidolina	Siphonophorae	Abylidae	Transcriptome	SRR871525
Agalma elegans	Hydrozoa	Hydroidolina	Siphonophorae	Agalmatidae	Transcriptome	SRR6512865; SRR6512866
Apolemia lanosa	Hydrozoa	Hydroidolina	Siphonophorae	Apolemiidae	Transcriptome	SRR6512854
Kephyes ovata	Hydrozoa	Hydroidolina	Siphonophorae	Clausophyidae	Transcriptome	SRR1548372
Cordagalma sp	Hydrozoa	Hydroidolina	Siphonophorae	Cordagalmatidae	Transcriptome	SRR1548356
Diphyes dispar	Hydrozoa	Hydroidolina	Siphonophorae	Diphyidae	Transcriptome	SRR6512850; SRR6512858; SRR6512859; SRR6512860;

						SRR6512861; SRR6512864; SRR6512867; SRR6512867
Erenna richardi	Hydrozoa	Hydroidolina	Siphonophorae	Erennidae	Transcriptome	SRR1548360
Forskalia asymmetrica	Hydrozoa	Hydroidolina	Siphonophorae	Forskaliidae	Transcriptome	SRR1548361
Hippopodius hippopus	Hydrozoa	Hydroidolina	Siphonophorae	Hippopodiidae	Transcriptome	SRR1548371
Physophora gilmeri	Hydrozoa	Hydroidolina	Siphonophorae	Physophoridae	Transcriptome	SRR6512853
Craseoa lathetica	Hydrozoa	Hydroidolina	Siphonophorae	Prayidae	Transcriptome	SRR871529
Bargmannia elongata	Hydrozoa	Hydroidolina	Siphonophorae	Pyrostephidae	Transcriptome	SRR1548343; SRR1548344; SRR1548345; SRR1548346; SRR1548347
Resomia ornicephala	Hydrozoa	Hydroidolina	Siphonophorae	Resomiidae	Transcriptome	SRR1548382
Rhizophysa filiformis	Hydrozoa	Hydroidolina	Siphonophorae	Rhizophysidae	Transcriptome	SRR1548383
Stephalia sp	Hydrozoa	Hydroidolina	Siphonophorae	Rhodaliidae	Transcriptome	SRR6512855
Craspedacusta sowerbii	Hydrozoa	Trachylinae	Limnomedusae	Olindiidae	Genome	GCA_003687565.1
Scolionema suvaense	Hydrozoa	Trachylinae	Limnomedusae	Olindiidae	Genome	SRR9613700
Liriope sp.	Hydrozoa	Trachylinae	Trachymedusae	Geryoniidae	Transcriptome	SRR3407335
Enteromyxum leei	Myxozoa	Myxosporea	Bivalvulida	Myxidiidae	Genome	GCA_001455295.2
Thelohanellus kitauei	Myxozoa	Myxosporea	Bivalvulida	Myxobolidae	Genome	GCA_000827895.1
Sphaeromyxa zaharoni	Myxozoa	Myxosporea	Bivalvulida	Sphaeromyxidae	Genome	GCA_001455285.1
Kudoa iwatai	Myxozoa	Myxosporea	Multivalvulida	Kudoidae	Genome	GCA_001407235.2
Polypodium hydriforme	Myxozoa	N/A	N/A	N/A	Transcriptome	GBGH01
Atolla vanhoeffeni	Scyphozoa	Coronamedusae	Coronatae	Atollidae	Transcriptome	SRR1952729
Periphylla periphylla	Scyphozoa	Coronamedusae	Coronatae	Periphyllidae	Transcriptome	SRR1915828
Cassiopea xamachana	Scyphozoa	Discomedusae	Rhizostomeae	Cassiopeidae	Genome	GCA_900291935.1
Nemopilema nomurai	Scyphozoa	Discomedusae	Rhizostomeae	Rhizostomatidae	Genome	GCA_003864495.1
Chrysaora achlyos	Scyphozoa	Discomedusae	Semaeostomeae	Pelagiidae	Genome	SRR7906160
Aurelia aurita	Scyphozoa	Discomedusae	Semaeostomeae	Ulmaridae	Genome	GCA_004194415.1
Craterolophus convolvulus	Staurozoa	N/A	Stauromedusae	Craterolophidae	Transcriptome	HAGZ01
Haliclystus sanjuanensis	Staurozoa	N/A	Stauromedusae	Haliclystidae	Transcriptome	HAHB01
Calvadosia cruxmelitensis	Staurozoa	N/A	Stauromedusae	Kishinouyeidae	Genome	GCA_900245855.1
Lucernaria quadricornis	Staurozoa	N/A	Stauromedusae	Lucernariidae	Transcriptome	HAHD01

Supplemental Table 2. Presence and absence of *Frizzled* genes in hydrozoans.

Species	Subclass	Assembly type	*frizzled1*	*frizzled2*	*frizzled3*	*frizzled4*	Colonial?
Hydractinia symbiolongicarpus	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Podocoryna carnea	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Hydra oligactis	Hydroidolina	Genome	Yes	Yes	**No**	Yes	**No**
Hydra viridissima	Hydroidolina	Genome	Yes	Yes	**No**	Yes	**No**
Hydra magnipapillata	Hydroidolina	Genome	Yes	Yes	**No**	Yes	**No**
Millepora complanata	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Turritopsis sp. SK-2016	Hydroidolina	Transcriptome	Yes	**No**	Yes	**No**	Yes
Porpita porpita	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Ectopleura larynx	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Clytia hemisphaerica	Hydroidolina	Genome	Yes	Yes	Yes	Yes	Yes
Dynamena pumila	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Abylopsis tetragona	Hydroidolina	Transcriptome	Yes	Yes	**No**	Yes	Yes
Agalma elegans	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Apolemia lanosa	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Kephyes ovata	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Cordagalma sp.	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Diphyes dispar	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Erenna richardi	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Forskalia asymmetrica	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Hippopodius hippopus	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Physophora gilmeri	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Craseoa lathetica	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Bargmannia elongata	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Resomia ornicephala	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Rhizophysa filiformis	Hydroidolina	Transcriptome	**No**	Yes	Yes	Yes	Yes
Stephalia sp.	Hydroidolina	Transcriptome	Yes	Yes	Yes	Yes	Yes
Craspedacusta sowerbii	Trachylinae	Genome	Yes	Yes	Yes	Yes	Yes
Scolionema suvaense	Trachylinae	Genome	Yes	Yes	Yes	Yes	**No**
Liriope sp.	Trachylinae	Transcriptome	Yes	Yes	Yes	Yes	**No**